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Response to "Comment on 'Application of an *in Vitro* Assay to Identify Chemicals That Increase Estradiol and Progesterone Synthesis and Are Potential Breast Cancer Risk Factors'"

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We appreciate the points raised by Burgoon and Borgert regarding our recent analysis of the U.S. Environmental Protection Agency's (EPA) data from the high-throughput (HT) H295R screening of 656 chemicals in concentration-response.[1] We considered these same points as we conducted our initial work and further analyzed this issue (complete details are available at https://github.com/SilentSpringInstitute/E2upP4up_followup_Burgoon). We disagree with their assertion that the 296 chemicals we found to increase estradiol (E2) and progesterone (P4) in the HT-H295R assay are mostly false positives.

In order to reach their conclusion about false positives, Burgoon and Borgert made two decisions that are inconsistent with standard approaches used by the U.S. EPA Endocrine Disruptor Screening Program[2] and the Organisation for Economic Co-operation and Development.[3] First, they did not normalize hormone concentration changes from treatment to concurrent plate-specific controls. Second, they required a hormone concentration to increase above a threshold they selected for the response to be positive. Neither the pooling of controls nor the application of a hormone concentration threshold is disclosed in their letter but are described in their GitHub code (https://github.com/DataSciBurgoon/toxcast_steroidogenesis/).

Burgoon and Borgert miscalculated the fraction of positives among all the chemicals that the U.S. EPA screened for two reasons. First, they excluded 84 chemicals tested in 2017, which we included, inflating the fraction positive. In addition, over 2,012 chemicals were initially tested at a single dose in HT-H295R, and 656 were selected for subsequent testing in concentration-response format because they affected multiple hormones. Thus, the 296 positive chemicals we identified as active are derived from screening 2,012 chemicals.

In response to comments from Burgoon and Borgert, we assessed reproducibility of the data by analyzing concordance among the 107 chemicals tested in replicate. We found many replicates (12 of 23 E2-up chemicals and 18 of 21 P4-up chemicals) showed concordant positive results. Lower concordance among E2-up chemicals is consistent with the overall lower dynamic range of E2 concentration changes in the assay.[1] Overall reproducibility among replicates—especially for P4—adds to the evidence that the data are reliable and that the standard practice of normalization to concurrent controls provides robust results.

Contrary to Burgoon and Borgert's assertions, we used raw data from the U.S. EPA's ToxCast database, although—as we made clear in our paper—we used the analysis of variance–based method described by Haggard et al.[4] to determine the chemical–hormone hit–call instead of using the ToxCast automatic data processing pipeline.[5] In addition, we mapped the chemicals we identified as increasing E2 or P4 onto data from the National Toxicology Program's Integrated Chemical Environment database, as Burgoon and Borgert suggested. After filtering for the HT-H295R assay, we found quality control flags for 22 chemicals (13 that increased E2, 4 that increased P4, and 5 that increased both). Most of the flags note low chemical concentration in the well, which can result in false negatives, but positive results are still qualitatively useful.

It is unclear why Burgoon and Borgert highlight "day-effects" only for P4 data and suggest that this is grounds for dismissing the P4 results entirely. Dimethyl sulfoxide controls are included on every plate to account for variability between measurements from different plates. In addition, good concordance between replicates for P4 indicates that this is not a major concern, and P4 results are even more reliable than those for E2.

Acknowledgments

Although our original analysis relied on hit–calls from the U.S. Environmental Protection Agency's (EPA) analyses of these data,[4,6] A. Borrel and J. Kay assisted us in conducting additional data analysis to respond to Burgoon and Borgerts's comments, and so we have included them as authors in this response. We also appreciate the support from R.W. Setzer (emeritus U.S. EPA) and K. Paul-Friedman (U.S. EPA) in preparing this response.

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